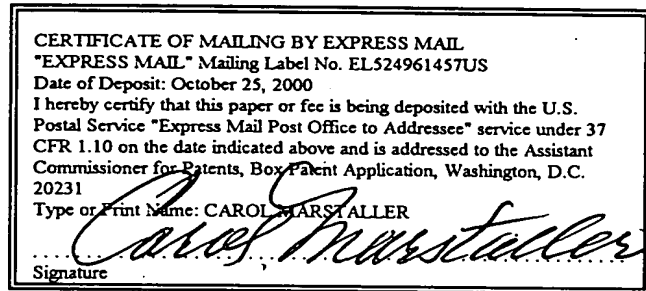


Patent Application  
Docket No. 48279-9USPT



**MICROBIAL EXOPOLYSACCHARIDE  
AND USES THEREOF**

**CROSS-REFERENCES TO RELATED APPLICATIONS**

This Application for Patent claims the benefit of priority from, and hereby incorporates by reference the entire disclosure of, co-pending U.S. Provisional Application  
5 for Patent Serial No. 60/161,588 filed October 26, 1999 and Serial No. 60/161,391, filed October 26, 1999.

**TECHNICAL FIELD OF THE INVENTION**

The present invention relates in general to a novel non-  
10 pathogenic microbe that produces a nontoxic, non-antigenic exopolysaccharide. The use of the microbe and exopolysaccharide in environmental engineering, agricultural, geologic, consumer and medical applications is described.

## BACKGROUND OF THE INVENTION

The invention pertains to a novel non-pathogenic microbe that produces a non-toxic, non-antigenic exopolysaccharide. The exopolysaccharide can be used as a biofilm in environmental engineering and agricultural applications and as a filler or polymer in consumer and medical applications. Biofilm applications are described first, then particular medical applications are described.

The term "biofilm" is used to describe an organic material that includes microorganisms embedded in a polymer matrix of their own making. The matrix consists largely of exopolysaccharides and is a tough, elastic, mucoidal material that adheres strongly to soil particles. Growth of a biofilm in a sandy soil is achieved by injecting a bacterial and nutrient solution into soil specimens. The resulting biofilm treatment is used to clog soil pores, thereby reducing the ability of the soil to transmit fluids.

Examples of biofilms are produced by certain strains of *Klebsiella pneumoniae* and *Pseudomonas* species. A problem with the use of *K. pneumoniae* is that *Klebsiella* is a genus that includes a number of human pathogens. Furthermore, the pathogenicity of *K. pneumoniae* itself is associated with its ability to create a mucoidal exopolysaccharide used in

attachment and colonization that helps the pathogen evade both the non-specific and specific immune clearing defensive mechanisms.

Another example of a biofilm is described in U.S. Patent  
5 No. 4,800,959, by Costerton, which discloses the use of a  
microbial process for selectively plugging a subterranean  
formation. In the process taught, a highly permeable stratum  
or zone in a subterranean reservoir is plugged using  
*Klebsiella* or *Pseudomonas* bacteria that were starved to  
10 reduce their size prior to being injected into the target  
zone. The bacteria regain full cell size, proliferate and  
commence production of biofilm-forming exopolysaccharides  
upon exposure to minimal nutrient containing media. The  
biofilm produced by these bacteria selectively seal off the  
15 high permeability zones of a formation and reduce aqueous  
flow through the zone.

In addition to the above described biofilm uses, there  
has been a need for perfusion solutions and blood  
substitutes. Currently available and approved compounds,  
20 however, have so far failed to meet the increasing demands  
on our blood provider system. A number of blood substitutes  
have been developed over the last few years to attempt to  
meet the increasing demand for blood, blood substitutes and

plasma expanders. Unfortunately, many of the plasma expanders that are currently in use fail as the small molecules on which they depend to provide osmotic pressure readily traverse capillary beds as a consequence of the negative osmotic pressure found in post-arterial capillary beds. The loss of osmotic potential, makes the long-term use of current plasma expanders for maintaining proper ionic or fluid balance or plasma volume in a mammalian subject unsatisfactory.

Those blood substitutes that have an impermeable substance to maintain volume use human serum albumin or a mixture of plasma proteins as the oncotic agent. These substitute plasma proteins depend on the same blood and plasma supply as our current blood provider system, therefore failing to meet the increased demand for these products.

A number of patents have issued to Segall that are directed to blood and plasma substitutes. U.S. Patent No. 4,923,442, and the reissue thereof, discloses a number of solutions used in blood substitution of living subjects all of which include at least some concentration of a cardioplegia agent, usually potassium ion. U.S. Patent No. 4,923,442 discloses surgical methods, particularly in respect to instrument placement and the control of pulmonary

wedge pressure generally applicable to perfusion of subjects.  
U.S. Patent No. 5,130,230 discloses a blood substitute that  
may be used as a system of solutions in which a number of  
solutions, are used sequentially to completely replace the  
5 blood of living subjects. U.S. Patent No. 5,130,230  
discloses that the blood substitute comprises "an aqueous  
solution of electrolytes at physiological concentration, a  
macromolecular oncotic agent, a biological buffer having a  
buffering capacity in the range of physiological pH, simple  
10 nutritive sugar or sugars, and magnesium ion in a  
concentration sufficient to substitute for the flux of  
calcium across cell membranes."

In addition to the patented inventions described above,  
a number of commercially available products have been used  
15 for the treatment of hypovolemic patients. These include:  
HESPANJ (6% hetastarch in 0.9% sodium chloride injection,  
PENTASPANJ (10% pentastarch in 0.9% sodium chloride injection  
[both by DUPONT PHARMACEUTICALS, Wilmington Del.]), MACRODEXJ  
(6% dextran 70 in 5% dextrose injection or 6% dextran 70 in  
20 0.9% sodium chloride injection [PHARMACIA, INC., Piscataway,  
N.J.]) and RHEOMACRODEXJ (10% dextran 40 in 5% dextrose  
injection or 10% dextran 40 in 0.9% sodium chloride injection  
[PHARMACIA, INC., Piscataway, N.J.]). All of these

products, however, depend on compounds that are polymeric and that often dissociate or are broken down by natural physiologic enzymes with time. Alternatively, bacteria may take advantage of these newly supplied nutrient sources, causing severe septicemia in patients that are infected by pathogens at the time of injury. Thus, a need remains for a better oncotic agent.

#### SUMMARY OF THE INVENTION

10       The newly discovered bacterium LAB-1, deposited at ATCC No. PTA-2500, possesses a number of potential commercial biofilm applications. These include, but are not limited to: (1) subsurface biofilm cutoff wall formation; (2) subsurface liners that include compacted, biofilm treated soil; (3) in-  
15       situ biofilm liners; (4) barriers made by treating geotextiles with biofilm materials; (5) improved ability of sand to retain moisture; (6) reclamation of poor soils and conversion into agriculture land; (7) significantly increased soil biomass in the form of polymers that function as a  
20       nutrient supply for plant growth and/or help retain nutrients and water; and (8) providing cohesion to otherwise cohesionless soils (such as sand dunes), thus making the soil more resistant to erosion by wind and/or water.

It has been found that the prior art methods and biofilms fail to provide biologically and environmentally safe and efficacious water, soil and waste retention characteristics. A significant problem with existing  
5 technology is the pathogenicity of the bacteria used to produce the biofilms. The present invention, therefore, is directed to a non-pathogenic bacterium that produces a biofilm made of exopolysaccharide that is essentially made of neutral sugars that migrate at the same rate as: mannose,  
10 fucose, fructose and galactose, acidic sugars that migrate at the same rate as fucose and amine sugars that migrate at the same rate as glucose and fucose.

More particularly, the bacterium is a LAB-1 strain. The biofilm producing bacterium may be further defined as being  
15 capable of growth between about pH 4 and 11 and between about 15° and 45°C. The LAB-1 strain is capable of growth in minimal growth media, or may be grown in an aqueous nutrient medium that includes yeast, peptone and mineral salt ingredients. LAB-1 is a gram-negative, rod-shaped bacterium  
20 of about 0.2 X 0.8  $\mu\text{m}$  that secretes the exopolysaccharide described herein.

In one embodiment of the present invention, the LAB-1 strain is used in plugging a permeable subterranean stratum

by providing LAB-1 bacteria in a nutrient-containing solution into the target stratum. The nutrient-containing solution is generally adapted to provide substantial and uniform growth conditions for the LAB-1. Sufficient biofilm is produced under these conditions to effectively plug the stratum. For example, the bacterium *in situ* can yield a saturated hydraulic conductivity equal to or less than  $1.5 \times 10^{-5}$  cm/sec, equal to or less than  $1.0 \times 10^{-7}$  cm/sec or even equal to or less than  $1.5 \times 10^{-8}$  cm/sec.

Alternatively, the bacteria may be preincubated in culture in an aqueous suspension medium with agitation for an incubation period sufficient to initiate bacterial exopolysaccharide production before injection into the stratum. The method of plugging the subterranean stratum may also include draining nutrient deficient suspension medium from the reservoir, and recharging the reservoir with aqueous nutrient medium to maintain bacterial growth for an elapsed time period sufficient to establish a biofilm of prescribed saturated hydraulic conductivity. The draining and recharging steps with aqueous nutrient medium may be conducted at least once every 48 hours of elapsed time period. The step of pre-incubating the bacteria may be, e.g., for at least about 72 hours. These growth conditions

permit for the establishment of a biofilm having a population between about  $10^5$  -  $10^{15}$  bacterial Colony Forming Units per square centimeter on a slide surface.

5 The biofilm may be used to plug open conduits, deposited in a subsurface biofilm cutoff wall, used to enhance the water retaining ability of subsurface liners or even for improving the water retention capabilities of compacted, semi-compacted or loosened biofilm treated soil. When used in a liner, the biofilm may be deposited *in-situ*. The  
10 biofilm may also be used along with and/or to enhance environmental barriers by treating geotextiles with the biofilm.

Another important aspect of this polymer is its lack of antigenicity and toxicity in an animal system. This suggests  
15 several consumer/medical applications, including: (1) use as a food additive or food thickening or filler agent; (2) use as plasma expander; (3) use in polymer industry; (4) use as chromatography matrix support for purification of chemicals; (5) use in scientific research as suspension solution instead  
20 of ficoll and the like; (6) use in determining the gene content of the organism, especially those coding for the biosynthesis of the exopolysaccharide polymer; (7) use of the polymer materials in the cosmetic field; (8) use to augment

insect or animal diets; (9) use as an additive in tissue culture media; (10) for use as a semi-solid to solid matrix, e.g, gel electrophoresis; (11) for use as an additive in toothpaste, ointments, creams and lotions; (12) for mixing  
5 with dyes, stains, paints and varnishes; (13) for inclusion in dialysis; (14) for use in composite materials, e.g., bricks, tile, mortars; (15) for use as part of a sealant; (16) viscosity modifier for oils, waxes & greases; (17) use as a filler, thickener or extender in pharmaceutical  
10 preparations; (18) use of the polymer in bioscaffolding applications, including wound-healing applications; and (19) use as a bacteriostatic (biostat) agent to inhibit or at least fail to support bacterial growth, and even possibly as a biocide.

15 In particular, a compound is needed for use as a plasma extender that serves to increase blood volume and that is impermeable at blood capillaries. The compound must not readily dissociate or be rapidly broken down by natural physiologic enzymes with time. Furthermore, the compound and  
20 its use as a plasma expander must not provide bacteria with an exogenous nutrient source, which may lead to accentuating already severe septicemia in patients that are infected by

pathogens at the time of the injury that is causing hypovolemia.

More particularly, the present invention is an exopolysaccharide produced by the LAB-1 bacterial strain. The  
5 exopolysaccharide does not appear to easily support bacterial growth. This was determined by testing the ability of *E. coli* or *B. indica* to grow on the exopolysaccharide and no growth was observed. Further, the exopolysaccharide is not antigenic as tested by injection  
10 into mice. Thus, the product appears to satisfy some of the basic parameters required for a plasma expander.

The exopolysaccharide is secreted into the cell culture medium and collected for use in, e.g., a plasma expander. When used as a plasma expander alone, or in combination with  
15 other elements, the exopolysaccharide will be provided in an isotonic solution. In one embodiment, a blood-free plasma expander and blood substitute for use in a subject in need thereof includes a single solution with at least two water soluble oncotic agents, one of which is a water soluble  
20 polysaccharide oncotic agent and one of which is serum albumin, wherein the exopolysaccharide consisting essentially of mannose, fucose, fructose and galactose, acidic fucose and amine containing glucose and fucose.

The plasma expander and blood substitute may have a ratio of water soluble exopolysaccharide oncotic agent to serum albumin between 1:1 and 1:2, weight to weight. The combined percentage of water-soluble exopolysaccharide oncotic agent and serum albumin in a solution of the plasma expander and blood substitute may be in the range of between about 4%-6% weight to volume.

The plasma expander and blood substitute may also include a number of cations, alone or in combination. For example, the cations may be provided in the following concentrations:  $\text{Na}^+$  at 110 to 120 mEq/l,  $\text{Ca}^{++}$  at about 5 mEq/l,  $\text{K}^+$  at 0 to 3 mEq/l, and  $\text{Mg}^{++}$  at 0 to 0.9 mEq/l. These cations may be supplied as dissolved chloride salts. The plasma expander and blood substitute may also include at least one buffer, for example, a lactate and/or bicarbonate buffer. When buffered, the plasma expander will generally be a biological buffer having a buffering capacity in the pH range of about 6.8 to 7.8.

When used in hypovolemic patients, e.g., those that have lost a large volume of blood due to trauma, additional agents may be included in the plasma expander to aid in recovery. Such agents may include, Vitamin K in a concentration of

about 1-4 mg/l, amylase, clotting factors, t-PA or even erythropoietin.

5 In non-medical uses, the exopolysaccharide of the present invention may be used as a chromatography matrix support for purification of chemicals. One such use will be as a suspension solution for use in centrifugation. The exopolysaccharide may even be used in solution as a suspension solution for use in size separation.

10 The present invention may also be used as a biologically stable, non-toxic material for use in coated plates for a number of biological and analytical uses. Examples of such uses include the coating of tissue culture plates for maintaining the growth, in vitro, of cells. Cells that may be grown on the surface of the exopolysaccharide include  
15 prokaryotic and eukaryotic cells. In an analytical setting, the exopolysaccharide disclosed herein may be used as a coating for instrumentation, such as biosensors, that require the maintenance of a biologically compatible environment.

20 A more complete appreciation of the present invention and the scope thereof can be obtained from the accompanying drawings which are briefly summarized below, the following detailed description of the presently-preferred embodiments of the invention, and the appended claims.

#### BRIEF DESCRIPTION OF THE DRAWINGS

A more complete understanding of the method and apparatus of the present invention may be obtained by reference to the following Detailed Description when taken  
5 in conjunction with the accompanying Drawings wherein:

FIGURE 1 is a photograph of Gram stained LAB-1 at 100X magnification.

FIGURE 2 is a Coomassie stained SDS-PAGE gel of the total protein content of LAB-1 and *B. indicia* grown on solid  
10 culture.

FIGURE 3 is a Coomassie stained SDS-PAGE gel of the total protein content of LAB-1 and *B. indicia* grown in liquid culture.

FIGURE 4 is the data from gas chromatography of fatty  
15 acids in LAB-1.

FIGURE 5 is a FACE gel showing the sugars identified in the exopolysaccharide produced by LAB-1. Lanes: 1 - MONO Ladder Standard 2 (100 pmol ea. monosaccharide); 2 - Amine hydrolysis reaction products; 3 [S] - MONO Ladder Standard  
20 2 (100 pmol ea. monosaccharide; scanned for trace shown in [S] Scan); 4 - Neutral hydrolysis reaction products; 5 - Sialic acid hydrolysis reaction products; 6 - NANA labeling

control 1 (100 pmol); 7 - MONO composition control; 8 - MONO Ladder Standard 2 (100 pmol ea. monosaccharide).

FIGURE 6 is a MALDI trace of the exopolysaccharide produced by LAB-1.

5        FIGURE 7 is a drawing of a cross sectional view of a barrier created with the biofilm of the present invention.

FIGURE 8 is a graph of the hydraulic conductivity versus time of a LAB-1 containing biofilm.

10        **DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EXEMPLARY EMBODIMENTS**

      The present invention will now be described more fully hereinafter with reference to the accompanying drawings, in  
15        which preferred embodiments of the invention are shown. This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully  
20        convey the scope of the invention to those skilled in the art.

**EXAMPLE 1 CHARACTERIZATION OF LAB-1 ORGANISM AND EXOPOLYSACCHARIDE**

Samples of LAB-1 have been deposited under ATCC No. PTA-2500. LAB-1 is a Gram-negative, rod-shaped bacterium isolated by the present inventor from constructed soil samples in the state of Wyoming. The soil sample was constructed by J. Turner at the University Wyoming and contained a contaminant in a background of a *Beijerinckia indica*. The contaminant, LAB-1, was isolated and studied because of its excessive slime production.

A variety of biochemical tests were performed in order to identify the species and the genus of organism. The tests included: assessment of growth conditions, culture appearance, cell appearance and staining characteristics, optimal temperature and pH growth range, oxygen requirements, antibiotic sensitivity testing, tests for catalase and oxidase (using TAXOS NJ disks), amino acid utilization as carbon source, nitrogen fixation, motility test, and a variety of commercially available tests from MICRO-IDJ, OXYFERMJ, ENTEROTUBEJ and BIOLOG GN MICROPLATEJ.

Because the organism was a contaminant in soil amended with a *B. indica* culture, *B. indica* and the LAB-1 organism were grown on plates and total protein profiles were compared by SDS-PAGE. Fatty acid analysis was performed by Microbial

ID (MIDI) of Newark, DE who used the high resolution MIDI  
SHERLOCK SYSTEMJ to identify fatty acids with high resolution  
gas chromatography. The same laboratory also sequenced the  
16S gene and compared it against the proprietary MICROSEQJ  
5 database (PE APPLIED BIOSYSTEMJ).

The exopolysaccharide was further analyzed by GLYKOJ  
(NOVATOJ, CA) fluorophore-assisted carbohydrate  
electrophoresis (FACE), and matrix-assisted laser  
desorption/ionization mass spectrometry (MALDI).  
10 Immunogenicity of the exopolysaccharide was also tested in  
mice.

Colonies of LAB-1 on solid medium were irregular with  
an undulate edge. They exhibited convex elevation, a smooth  
glistening surface, were white in color and translucent to  
15 opaque. The consistency of the colonies was that of a very  
tenacious and elastic slime. The LAB-1 exopolysaccharide  
exhibits tremendous tenacity, extending without breakage when  
pulled with a glass rod over a foot. Due to the production  
of this exopolysaccharide, it was found difficult to lift the  
20 colonies from agar plates.

AB13 culture medium may be used to grow the LAB-1 strain  
and is made as follows: Per liter of water add 20 g glucose,  
1 g NaCl, 1 g yeast extract or 2 g NaNO<sub>3</sub>, 8 g K<sub>2</sub>HPO<sub>4</sub>, 0.2 g

$\text{KH}_2\text{PO}_4$ , 0.5 g  $\text{MgSO}_4$ , and 150  $\mu\text{l}$  5%  $\text{FeCl}_2$ . To make solid medium, 15 g of agar are added to the medium. This simplified end medium makes large-scale production of the polysaccharide polymer affordable.

5        Growth in liquid AB13 medium was perfuse and the turbidity was dense and uniform. When the LAB-1 bacterium is grown in liquid medium, the entire solution becomes very viscous with a consistency ranging from that of heavy corn syrup to that of egg whites, depending on the stage of  
10 growth. Cultures grown without shaking showed a flocculent deposit along with smooth surface growth. The cultures had a very distinct odor, somewhat sweet smelling, but not pleasant.

      The bacteria were bacilliary with rounded ends and  
15 parallel sides and were determined to be Gram negative. There were some irregularities observed in the cell population in cell size, due mainly to length differences, but the average size was about 0.2 X 0.8  $\mu\text{m}$ . The  
arrangement of cell packets seemed to be irregular, although  
20 a large percentage of cells were aligned side-to-side. The clumping of cells was believed to be due to the tenacious slime layer. The exopolysaccharide could be seen in Gram staining as a light cloud surrounding the cells. FIGURE 1

shows the Gram stained cells at 100X. The spore stain showed only red vegetative cells, no spores were observed. The capsule stain showed no capsules, but rather an indefinite exopolysaccharide surrounding the cells. Observation of the stab culture showed that the organism was motile.

Colony morphology on plates grown at 26°, 30°, 37°C and 45°C was the same, although growth was optimal at 37°C. Elasticity of the slime layer was also unchanged. Growth was unchanged in liquid AB13 cultures ranging from pH 4, 9 and 11. Growth was not observed at pH 2. Colony morphology on plates was also unchanged over this pH range. The LAB-1 organism was determined to be a facultative anaerobe.

Various biochemical test results are summarized in the following tables:

Table 1. Morphology of LAB-1

Morphological Characteristics	Results
Gram reaction	negative
Cell Shape	bacilli
Spores	none
Growth temp.	26-37°C
pH range	4-11
Growth in peptone	+
Motility	+

Table 2. Antibiotic Profile of LAB-1

Antibiotic Susceptibility	Results
Amikacin	sensitiv e
Ampicillin	resistan t
Erythromycin	resistan t
Neomycin	sensitiv e
Novobiocin	sensitiv e
Penicillin	resistan t
Polymyxin B	sensitiv e
Streptomycin	resistan t
Taxos A	resistan t
Tetracycline	sensitiv e

Table 3. Biochemistry of LAB-1

	Biochemical Properties	Results
	Adonitol fermentation	+
5	Ammonification	+
	Arabinose fermentation	+
	Arginine dihydrolase	+
	Beta-galactosidase	-
	Catalase	+
10	Citrate utilization	+
	Cytochrome oxidase	-
	Esculin hydrolysis	-
	Glucose fermentation	+
	Aerobic glucose	+
15	Anaerobic glucose	+
	Hydrogen sulfide	-
	Indole (tryptophanase)	-
	Inositol fermentation	+
	Lactose fermentation	+
20	Lysine decarboxylase	-
	Malonate utilization	-
	Maltose oxidation	+
	Mannitol oxidation	+
	N <sub>2</sub> gas production	-
25	Nitrate reductase	-
	Nitrogen fixation	-
	Ornithine decarboxylase	-
	Phenylalanine deaminase	-
	Sorbitol fermentation	+
30	Sucrose oxidation	-
	Urease	+
	VP	+
	Xylose oxidation	+

Table 4. Carbon Utilization of LAB-1

	Carbon Utilization	Resu lt	Carbon Utilization	Resu lt
	A-cyclodextrin	+	Itaconic acid	-
5	Dextrin	+	A-keto-butyric acid	-
	Glycogen	+	A-keto-glutaric acid	-
	Tween 40	+	A-keto-valeric acid	-
	Tween 80	+	D, L-lactic acid	+
	N-acetyl galactosamine	+	Malonic acid	+
10	N-acetyl-D-glucosamine	+	Propionic acid	-
	Adonitol	+	Quinic acid	+
	L-arabinose	+	D-saccharic acid	+
	D-arabitol	+	Sebacic acid	-
	D-cellobiose	+	Succinic acid	+
15	I-erythritol	-	Bromo Succinic acid	+
	D-fructose	+	Succinamic acid	+/-
	L-fucose	+	Glucuronamide	+
	D-galactose	+	Alaninamide	+/-
	Gentiobiose	+/-	D-alanine	+
20	A-D-glucose	+/-	L-alanine	+
	M-inositol	+	L-alanyl-glycine	+
	A-D-lactose	+	L-asparagine	+
	Lactulose	+	L-aspartic acid	+
	Maltose	+	L-glutamic acid	+
25	D-mannitol	+/-	Glycyl-L-aspartic acid	+
	D-mannose	+	Glycyl-L-glutamic acid	+
	D-melibiose	+	L-histidine	+
	B-methyl-d-glucoside	+	Hydroxy-L-proline	+
	D-psicose	+	L-leucine	-
30	D-raffinose	+	L-ornithine	-
	L-rhamnose	+	L-phenylalanine	-
	D-sorbitol	+	L-proline	+
	Sucrose	+	L-pyroglutamic acid	-
	D-trehalose	+	D-serine	+
35	Turanose	+	L-serine	+
	Xylitol	+	L-threonine	-
	Methyl pyruvate	+	Carnitine	-
	Methyl succinate	+	G-amino Butyric acid	+
	Acetic acid	+	Urocanic acid	-
40	Cis-aconitic acid	+	Inosine	+

	Citric acid	+	Uridine	-
	Formic acid	+	Thymidine	+
	D-galactonic acid	+	Phenylethylamine	-
	lactone			
5	D-galacturonic acid	+	Putrescine	-
	D-gluconic acid	+	2-amino ethanol	-
	D-glucosaminic acid	+	2,3-butanediol	-
	D-glucuronic acid	+	Glycerol	+
	A-hydroxybutyric acid	-	D,L-A- glycerol	+
			phosphate	
10	B-hydroxybutyric acid	+	Glucose-1-phosphate	+
	G-hydroxybutyric acid	-	Glucose-6-phosphate	+
	P-hydroxyphenyl acetic	+		
	acid			

15           Bacteriological information was compiled into the chart shown in Tables 1 through 4. This information was used to search the 9<sup>th</sup> edition (1994) of *Bergey's Manual of Determinative Bacteriology*. The Bergey's profiles of all Gram negative facultative anaerobic bacilli were researched

20           in an attempt to find potential matches. Special attention was paid to members of the genus *Klebsiella* because this was the closest match provided by BIOLOGJ, and, to the genus *Beijerinckia* because LAB-1 was isolated as a contaminant amongst a lawn of *Beijerinckia* colonies.

25           Although the information contained in Tables 1-4 was considered valuable, this particular method of exploration was quickly proven to be inconclusive. No exact matches

were identified, and, the partial matches found were too numerous to consider them all. Therefore, other more concrete methods of identification were utilized.

Because it was entirely possible that LAB-1 could have  
5 been a *Beijerinckia* species, protein profiles of each organism were compared. For the gel in FIGURE 2, the colonies of each organism were harvested from AB13 plates and sheared by vortexing with glass beads for five minutes. One volume of running buffer and one volume of bromophenol  
10 blue tracking dye was added to the lysate, and the result boiled for five minutes. After a brief spin, the samples were overlaid twice, with powdered sucrose to remove cell debris and 100  $\mu$ l were loaded onto a 4.5% stacking, 10% resolving polyacrylamide gel and run at 30-35 mAmp.

15 Samples used for FIGURE 3 were grown in liquid AB13 culture. One ml was harvested by pelleting for five minutes, washed with 1% NaCl and again with dH<sub>2</sub>O. Pellets were resuspended in 180  $\mu$ l of 50 mM Tris, and 10 mM EDTA, pH 8.0. Sixty  $\mu$ l of PSS (4X PSS is 700  $\mu$ l of 1 M Tris-HCl, pH  
20 6.8, 4.3 g sucrose, 4 mg bromophenol blue, 1.45  $\mu$ l of 20% SDS, 44  $\mu$ l of 0.5 M EDTA, 10 mg DTT and dH<sub>2</sub>O to 10 ml) were added to the cells and the mixture was boiled for five

minutes. Samples were overlaid with powdered sucrose to remove cell debris and run as above.

The Coomassie blue stained SDS-PAGE gels shown in FIGURES 2 and 3 clearly demonstrate that there are  
5 dissimilarities between the two organisms. It was concluded that the unknown organism LAB-1 was not a *Beijerinckia* species.

Fatty acid analysis by gas chromatography by MIDI LABSJ, Newark, DE, was employed to identify the organism.  
10 The growth conditions were standardized, the MIDI SHERLOCK SYSTEMJ was fully automated, and the data compared against proprietary databases containing the fatty acid profiles of more than 1,900 bacteria. The results are shown in FIGURE 4. The best match, *Kluyvera cryocrescens*, had a similarity  
15 of 0.835. This match would have been considered very good if there had been a separation of at least 0.100 between this first choice and the second choice, *Enterobacter taylorae*, with a similarity of 0.753.

Using these two species, reference was made to the 9<sup>th</sup>  
20 edition (1994) of *Bergey's Manual of Determinative Bacteriology* to compare their characteristics with those listed in Tables 1-4. Also compared was the third choice, *Kluyvera ascorbata*. There were many key differences in the

biochemical profiles of LAB-1 and each of the three matches.  
The properties found to be different amongst these four  
organisms are shown in Table 5. This information, along  
with the fatty acid analysis results, led to the conclusion  
5 that the organism was not a member of the genus *Kluyvera*.

Table 5 Differences between LAB-1 and *K. cryocrescens*, *K. ascorbata*, *E. Taylorae*

	LAB-1	<i>K. cryo.</i>	<i>K. ascorb.</i>	<i>E. Taylorae</i>
Adonitol fermentation	+	-	-	-
Arginine dihydrolase	+	-	-	+
Esculin hydrolysis	-	+	+	+
Indole (tryptophanase)	-	+	+	-
Inositol fermentation	+	-	-	-
Lactose fermentation	+	+	+	-
Lysine decarboxylase	-	-	+	-
Melibiose fermentation	+	+	+	-
MR	-	+	+	-
VP	+	-	-	+
Nitrate reductase	-	+	+	+

Ornithine decarboxylase	-	+	+	+
Raffinose fermentation	+	+	+	-
Sorbitol fermentation	+	+	+	-
Urease	+	-	-	-

The next logical step was to determine the 16S rRNA  
10 gene sequence. This technique currently is the method of  
choice for identification purposes. The gene sequence is  
shown in SEQ ID NO: 1. The identification based on the 16S  
rRNA gene sequence was determined to be *Leclercia*  
*adecarboxylata*. The difference in sequence homology between  
15 LAB-1 and *L. adecarboxylata* was only 0.59%. Stackebrandt &  
Goebel, INT'L J. SYSTEM. BACTERIOL. 44: 846 (1994) would  
consider this a species level match, however the confidence  
limits of the data obtained by MIDI LABSJ allowed  
identification only at the genus level. When the  
20 biochemical characteristics of LAB-1 and of *L.*  
*adecarboxylata* (9<sup>th</sup> edition Bergey's Manual of Determinative  
Bacteriology 1994) are compared, there are yet again,  
numerous differences. The differences led to the questions  
regarding this method.

Table 6 Differences between LAB-1 and *L. adecarboxylata*

	LAB-1	<i>L.</i> <i>adecarb.</i>
Arginine dihydrolase	+	-
Citrate utilization	+	-
Esculin hydrolysis	-	+
Glycerol utilization	+	-
Indole (tryptophanase)	-	+
Inositol fermentation	+	-
Malonate utilization	-	+
MR	-	+
VP	+	-
Nitrate reductase	-	+
Sorbitol fermentation	+	-
Urease	+	-

Because the same laboratory (MIDI LABSJ) produced two different identifications, the validity of both identities was suspect. However, 16S rRNA gene sequencing is considered to be the most reliable method currently available, and, therefore, the identification of *Leclercia adecarboxylata* must be further investigated. With all of the discrepancies that have been encountered, it is reasonable to conclude that this unknown organism (LAB-1) has not been previously identified.

Analysis of the exopolysaccharide produced by LAB-1 was by FACE (sugar content) and MALDI (size). Judging from the intensity of the bands shown on the FACE gel in FIGURE 5, the amount of fucose present is approximately twice that of

galactose; the amount of glucose is approximately 2.5 times that of galactose; and, the amount of mannose is approximately 3 times that of fucose. Therefore, the ratio of galactose:fucose:glucose:mannose is approximately 1:2:3:6. Further investigations regarding the nature and type of linkages and molecular weight determination of the polymer may be undertaken, as will be known to those of skill in the art.

Results of the MALDI study of the exopolysaccharide are shown in FIGURE 6. The data indicates that polymerization and depolymerization of the polysaccharide occurred readily as evidenced by the large range of molecular weights found. All monosaccharides identified were neutral sugars that migrate at the same rate as: mannose, fucose, fructose and galactose, acidic sugars that migrate at the same rate as fucose and amine sugars that migrate at the same rate as glucose and fucose. All are six-carbon sugars. This fact makes it impossible to determine the composition of the polymer when only the molecular weight is known. Those substances with molecular weights below 180 are likely breakdown products of the polymer. The largest polymer, molecular weight of 1066.38, was comprised of approximately six 6C sugars.

It was later discovered that the initial exopolysaccharide sample analyzed for immunogenicity in mice contained residual amino acids. The small amount of protein present was from the yeast extract in the media and from  
5 dead cells. Even with these amino acids present, it was determined that the exopolysaccharide was non-immunogenic, although a very small immunogenic reaction was observed. This reaction made it necessary to further purify the exopolysaccharide so that it was protein-free.

10 It was determined that the best way to free the exopolysaccharide of amino acids was to (i) modify the medium to contain no amino acids and (ii) limit growth of the organism to prevent cell death and breakdown. After implementing these two modifications, the exopolysaccharide  
15 was found to be free of protein (not shown). The protein-free exopolysaccharide was provided to WASHINGTON BIOTECHNOLOGY [St Louis, MO] to perform more in-depth studies to determine immunogenic properties. The results were gratifying and the exopolysaccharide immunized rabbits maintained antibody  
20 titers of less than 1:100, or non-detectable, for the entire 12 week experiment. In contrast, the inactivated cell-immunized rabbits reached antibody titers as high as 1:1,638,400. These results are very encouraging because a

non-immunogenic biopolymer with tremendous elasticity such as the one characterized in this study probably has numerous industrial, agricultural and biomedical applications.

Much information has been gathered about LAB-1.  
5 Unfortunately, its complete identity remains indeterminate. Reliable identification methods have been employed but the results do not agree with each other. It is highly likely that this organism has not been previously identified.

More research is warranted before a definitive  
10 identification can be made. Further studies should include direct comparison of LAB-1 with *Klebsiella*, *Kluyvera cryocrescens* and *Leclercia adecarboxylata*. Methods important to compare these organisms include protein profile determinations and DNA analysis. Results obtained from  
15 these approaches will provide good evidence of any phylogenetic relationships.

To further characterize the exopolysaccharide, the monosaccharide linkages and branching of the polysaccharides should be determined. Also, it would be very useful to  
20 determine the nature of its overall polymerization. The localization of the gene(s) coding for the polysaccharides may be determined, as will be known to those of skill in the art of molecular biology. Even further studies may be

conducted to identify LAB-1, and may include: chromosomal DNA fingerprinting, random primer PCR profiling, rRNA or other gene sequencing, determination of the G+C % content, lipid analysis and BioLOGJ analysis (a more comprehensive  
5 biochemical analysis). Also, detailed studies regarding the chemistry of the polysaccharide will be completed.

#### EXAMPLE 2 APPLICATIONS TO ENGINEERED WASTE CONTAINMENT AND TREATMENT

The LAB-1 strain may be used to construct environmental  
10 biofilm barriers for containment and treatment of contaminated soil and groundwater. The purpose of containment barriers is to control the transport of chemical contaminants from waste disposal facilities or from areas which have become/ contaminated by spills, industrial  
15 processes, illegal dumping or other sources. Several different types of barriers are possible, including the following: (1) subsurface biofilm cutoff wall; (2) subsurface liners consisting of compacted, biofilm treated soil; (3) in-situ biofilm liners; and (4) barriers  
20 made by treating geotextiles with biofilm.

The results disclosed herein demonstrate that soil hydraulic conductivity (k) may be reduced by several orders of magnitude by the addition of the biofilm-producing

bacterium disclosed herein. The reductions of  $k$  obtained using the LAB-1 strain are sufficient to meet Environmental Protection Agency (EPA) criteria for barrier materials, defined as a  $k$  value of  $10^{-7}$  cm/sec or less. The low  
5 hydraulic conductivity persists when the soil is permeated with a variety of chemical solutions, suggesting that a biofilm barrier may be compatible with a wide range of contaminants. The biofilm disclosed herein may also be useful for controlling contaminant transport mechanisms,  
10 such as diffusion, adsorption and biodegradation.

Solutions of biofilm and nutrient are pumped into the subsurface through a series of closely-spaced vertical wells. Formation of biofilm in the soil around the wells causes a decrease in soil permeability and decrease in  
15 contaminant transport sufficient to form an engineered barrier to contaminant migration. Specific design parameters such as well depth and spacing, pumping pressures, composition of bacterial and nutrient solutions, and time of pumping, are site-specific and depend upon site  
20 geology, type and extent of subsurface contamination, ground water conditions, and other variables which must be considered on a case-by-case basis.

FIGURE 7 shows one use of the present invention for the formation of subsurface liners for the containment of wastes in engineered disposal facilities, such as landfills. A landfill 10 is depicted in cross-sectional view. Waste 12 is disposed within a subsurface liner 14. If the liner 14 is being placed during the creation of the landfill 10, a biofilm liner may be used prior to deposition of the liner 14. In addition, a containment wall may be erected that surrounds the waste site, and additional layers of decontaminating biofilm barriers may be included.

In preexisting landfills, such as the one depicted in FIGURE 7, waste may leach in the form of a leachate 16 into subsurface strata 18 and 20. A biofilm barrier wall 28 is created that surrounds the waste 12 and captures the leachate 16. The biofilm barrier wall 28 is constructed so as to reach into strata 22, 24 into which the waste 12 does not leach. One advantage of the biofilm of the present invention is that it permits such remedial application to existing landfills that may be leaking and even prevents leachate 16 from reaching a subterranean water layer 26.

Current technology for a biofilm barrier wall 28, for example, may employ fine-grained soils that are field compacted to achieve a hydraulic conductivity of less than

10<sup>-7</sup> cm/sec (commonly referred to as "clay liners"). At many sites, such soil is not readily available and must be transported from off-site, increasing substantially the cost of compacted soil liners. Using the LAB-1 strain of the  
5 present invention soil, containment conditions may be met by treating readily available soils with the biofilm in order to achieve the low hydraulic conductivity required for compacted soil liners.

One specific field of use for the LAB-1 biofilms is  
10 creating subsurface biofilm liners that include spreading untreated soil in loose (uncompacted) lifts using conventional soil spreading equipment (e.g., bulldozers). Loose lifts will generally be 150 to 225 cm thick. A solution that includes water, LAB-1, and nutrients are  
15 applied to the soil, using, e.g., conventional equipment used to apply water to soil (e.g., a truck-mounted water tank with sprinkler hoses). The soil is then compacted using conventional equipment (e.g., sheeps foot rollers) to achieve the specified density, typically resulting in a  
20 compacted lift thickness of 100 to 150 cm. The required number of lifts and liner total thickness are site-specific design parameters which are determined by analysis of

contaminant transport and regulatory requirements for containment.

Compacted clay liners typically range from 0.6 to 1.3 meters thick. The proposed procedure is similar to field construction of clay liners, except that the soil is treated with a biofilm-producing solution. Alternatively, solutions of strain LAB-1 and nutrients are injected into the ground at a specified depth to create *in situ* biofilm liners. This type of liner is particularly useful at sites contaminated by accidental spills. Alternatively, previously grown biofilm may be mixed directly into or onto the soil.

Subsurface liners may also be constructed by treating geotextiles with biofilm. Geotextiles are generally made of synthetic fibers that are either woven or matted together, yielding a porous fabric that is used for soil separation, reinforcement, filtration or drainage. Containment barriers can be created by spraying bitumen, rubber-bitumen or other polymeric mixtures into a properly deployed geotextile that contains the LAB-1 produced biofilm disclosed herein. One particular example for use of the LAB-1 biofilm is in the application of a liquid solution containing strain LAB-1 and nutrients to geotextiles to clog the pore spaces and reduce permeability, creating a barrier to flow.

**EXAMPLE 3 MATERIALS: SOIL AND BACTERIA**

Soil used by the present inventors to analyze waste containment capability is a naturally occurring, easily attainable sand. Based on its grain size distribution and Atterberg limits, this soil is classified as SM, or silty sand of low plasticity, in the Unified Soil Classification System. Permeability tests yield a saturated hydraulic conductivity ( $k$ ) of approximately  $1.5 \times 10^{-5}$  cm/sec when compacted to maximum dry density. This value of  $k$  would make the soil unsuitable for use as a waste containment barrier. Initial studies indicated that  $k$  could be reduced to values on the order of  $10^{-8}$  to  $10^{-7}$  cm/sec, which is in the range required for waste containment, by treating this soil with the biofilm-producing bacterial strain LAB-1.

The operational procedure for use of the LAB-1 bacterium to form a biofilm that may be used to test water permeability may include the following steps: (1) compacting soil into a cylindrical specimen which is placed in a flexible wall permeameter, (2) permeating the specimen with a solution containing LAB-1, and (3) measuring the soil hydraulic conductivity while the specimen is permeated first with nutrient solution, then by water, as is taught by Dennis M.L. and Turner, J.P. J. GEOTECHNICAL & GEOENVIRONMENTAL

ENG. 124: 120-127 (1988) (in which a similar procedure was used with the bacterium *B. indica*).

Using the biofilm produced by the LAB-1 strain disclosed herein, hydraulic conductivity was reduced from  $k = 1.5 \times 10^{-5}$  to approximately  $k = 5 \times 10^{-8}$  cm/sec upon establishment of a plugging biofilm, which required permeation with nutrient solution for approximately one week. Most of this decrease occurred within 1 to 2 days, during which the  $k$  was reduced to less than  $10^{-7}$  cm/sec.

FIGURE 8 is a graph that shows hydraulic conductivity versus time for a specimen treated with LAB-1. The low hydraulic conductivity persisted for over 160 days, even though the nutrient solution was discontinued after 6 days.

#### EXAMPLE 4 APPLICATIONS TO AGRICULTURE

Large areas of the earth include desert lands that are not arable without large-scale reclamation. Reclamation in the context of desert lands requires not only irrigation, but extensive soil modification. The economical and social impact of successfully converting non-productive desert land into productive agricultural land is enormous and provides significant benefits to mankind.

The use of LAB-1 as a biologically and environmentally sound source of support and nutrients for soil treatment improves the agricultural properties of sandy soils as described herein. Many naturally existing desert soils are  
5 aeolian (wind-deposited) and consist of sand and silt sized particles with little or no organic content. Such soils are considered poor for agricultural development because they are highly porous, which promotes rapid infiltration and seepage of irrigation water away from the surface where it  
10 is most needed for crops. Lack of organic material generally corresponds to low nutrient content. Many desert areas are active aeolian environments in which wind is the dominant agent of sediment transport.

Agricultural development is severely impacted when  
15 topsoil is eroded and transported by wind. Considering the characteristics of sandy desert soils versus the requirements of soils for agriculture, the present invention includes the use of the LAB-1 derived biofilm for the treatment of agricultural soils to improve the following  
20 soil agricultural properties: (1) improved water retention characteristics; (2) enhanced ability to establish and support plant growth; and (3) improved erosion resistance. These improvements may be obtained by adding complete or

dried and pulverized biofilm, or by the application of LAB-1 strain in bacterial/nutrient solutions using conventional soil watering equipment (e.g., a truck-mounted tank with sprinkler hoses or conventional irrigation systems).

5           The biofilm of the present invention has been used for the treatment of soil. The biofilm altered the soil's properties in many ways that enhanced the soil's ability to support agriculture. These include the following: (1) an improved ability of sand to retain moisture; (2) an  
10   increased biomass in the form of polysaccharides that function as a nutrient supply for plant growth; (3) improved soil cohesion; and (4) increased resistance of soil to erosion.

          While this invention has been described in reference to  
15   illustrative embodiments, this description is not intended to be construed in a limiting sense. Various modifications and combinations of the illustrative embodiments, as well as other embodiments of the invention, will be apparent to persons skilled in the art upon reference to the  
20   description. It is therefore intended that the appended claims encompass any such modifications or embodiments. All references cited herein are hereby expressly incorporated by reference.